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Pre-clinical validation study of a miniaturized electrochemical immunoassay based on square wave voltammetry for early detection of carcinoembryonic antigen in human serum

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ABSTRACT

Background: The ELISA format for measuring carcinoembryonic antigen (CEA) serves as a reference standard against which other assays are compared. Because the World Health Organization (WHO) increasingly recommends the use of serum CEA as a diagnostic tool for cancer, it is relevant to explore the reliability of the new decentralized CEA point-of-care-testing (POCT) technologies that are available to physicians and patients, in compliance with mandates of the clinical laboratories' regulatory agencies.
Methods: Electrochemical immunoassay (ECIA) based on trace lead (Pb) analysis by anodic stripping techniques using sandwich-type immunocomplex conjugates: $^{MB}Ab/Ag_{CEA}/Ab^{PbS}$, and a commercial ELISA test system with optical transmission.
Results: The ECIA provides better analytical performance than does the ELISA. The within assay precision coefficient of variance ($\%CV_w$) of the ECIA is lower than the value recommended by the Hong Kong Association of Medical Laboratories (HKAML), and the recoveries of CEA at 1.0, 5.0, 10.0, 25.0 and 50.0 ng/ml are in the range of 99–110% for control serum samples. The ECIA showed a minimal positive bias of 0.0267 ± 0.3270 ng/ml ($P = 0.9389$).
Conclusions: The proposed CEA screening technology can be practically employed for decentralized clinical analysis of CEA in human serum. Therefore, it can be viewed as a control method for personalized therapy.

1. Introduction

Recently, the need for reliable diagnostic tests that can be used in the rapid detection of tumor markers in human serum has attracted the efforts of the scientific community [1]. Methods and strategies based on biochemistry, immunology and molecular biology have been developed and used in the determination of carcinoembryonic antigen (CEA) in human serum. Immunoassay techniques have become the dominant test methods for the clinical quantitative detection of tumor markers because of the highly specific molecular recognition of antigenic epitopes by antibodies. These techniques include radioimmunoassays [2–5], enzyme-linked immunosorbent assays (ELISA) [6,7], fluoroimmunoassays [8–10], chemiluminescent immunoassays [11–13] and electrochemiluminescent assays [14]. Although these detection techniques provide advantages (*e.g.*, sensitivity, precision and selectivity), they often suffer because they present radiation hazards, are time consuming, are expensive, and require qualified personnel and sophisticated instrumentation. Compared with traditional immunoassay methods, electrochemical immunosensors are specific, simple and convenient, and they offer multitarget analyses and miniaturization. The importance of miniaturizing an assay extends far beyond just increasing assay sensitivity or speed. The ability to carry out immunoassays in extremely small volumes can be very important in situations where minimizing the sample volume is absolutely necessary such as in the testing of neonates or critically ill patients, or in the analysis of physiologically restricted sites such as the brain, eye, or spine [15]. Electrochemical immunosensors can perform *in situ*, real-time, and automatic detection. Furthermore, their characteristics can satisfy the requirements for point-of-care testing (POCT) and bring molecular diagnostic assays to community health-care systems and underserved populations. However, devices used for the POC detection of protein biomarkers must be sensitive, robust, simple to operate and low cost. For successful clinical application, they must also be accurate for both normal and elevated levels of target protein concentrations, and they must be resistant to interference from non-targeted proteins. Such systems could enable rapid and inexpensive cancer testing in decentralized and under-resourced settings. New

Abbreviations: Ag, Antigen; CEA, Carcinoembryonic antigen; ECIA, Electrochemical immunoassay; HKAML, Hong Kong Association of Medical Laboratories; ^{MB}Ab, Magnetic bead conjugated to primary anti-CEA; ^{PbS}Ab, Lead sulfide conjugated to secondary anti-CEA; SPCE, Screen-printed carbon electrode; SWV, Square wave voltammetry

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technologies using nanostructured materials such as magnetic particles, gold nanoparticles, quantum dots, and carbon nanotubes are being developed to increase the sensitivity of electrochemical detection of cancer biomarkers [16]. The low detection limits that are achievable by such methods could facilitate the early detection of cancer and offer greater diagnostic accuracy for personalized therapy [17–19].

The use of magnetic beads (MBs) in the development of diagnostic devices is gaining popularity. For example, MBs have been used as substrates for the capture antibodies or for target antigens in immunoassays and enzyme-linked immunoassays [20–25]. MBs have more rapid reaction kinetics compared with bulk-solid surfaces, a high surface area per unit volume (because of their small diameter), and good stability [26]. Moreover, the relative ease of surface modification with functional groups, DNA, enzymes, or antibodies greatly contributes to the utility of beads in the development of sensitive and rapid electrochemical immunoassay systems [27,28]. Up to now, only a few electrochemical studies have been developed [29, and references cited therein], but scarcely, they have been validated for their use in clinical diagnosis. In addition, they have not considered the possibility to reduce the sample volume with miniaturized instrumentation and accessories, which could be an asset to integrate the advantageous properties of MBs and quantum dots in electrochemical immunoassays.

2. Materials and methods

2.1. Reagents

All stock solutions were prepared using deionized water type I ($\rho = 18.2$ MΩ cm; TOC ≤ 10 ppb) supplied from a Simplicity water dispenser system, EMD Millipore. The following materials were obtained from the indicated suppliers and used as received: BupH™ phosphate buffered saline (PBS) Packs (0.1 mol/l Na_3PO_4, 0.15 mol/l NaCl, pH 7.2), BupH™ 2-(N-morpholino)-ethane sulfonic acid (MES) Buffered Saline Packs, 2% bovine serum albumin (BSA) and Tween 20 were from Thermo Scientific. Purified native human CEA protein (30-AC25) (>80% pure by SDS-PAGE), monoclonal CEA antibody and monoclonal CEA antibody were from Fitzgerald Industries International, Inc. Carboxyl-modified magnetic beads (Dynabeads® M-270 carboxylic acid) were from Invitrogen. Lead nitrate, mercury standard solution ($[Hg^{2+}] = 1000$ μg/ml) and sodium acetate, anhydrous, were from J.T. Baker. Thioglycolic acid, thioacetamide, sodium hydroxide, acetic acid (glacial), hydrochloric acid (37%), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC) and N-hydroxysuccinimide (NHS) were from Sigma-Aldrich. Carcinoembryonic antigen (CEA) Test System was from AccuBind ELISA Microwells MonoBind®. Note: The calibrators for the CEA Test System are based in human serum. These were calibrated using a reference preparation that was assayed against the 1st International Reference Preparation (IRP # 73/601) [30].

2.2. Apparatus

A Thermo Electron Corporation microcentrifuge, IEC MicroECL was used in the conjugation–purification step. The magnetic bead-based immunoassay was conducted on a Biomerieux biomagnetic processing platform. NucliSens® MiniMAG™ was used for both immunoreaction mixing and separation. Square wave voltammetric measurements were performed on an electrochemical work station that includes a portable bipotentiostat μ-Stat, 200 (DropSens) and 3-electric contact connectors for a single working electrode, DSC (DropSens). Screen-printed carbon electrodes (SPCE) DS-110 (DropSens) were used in all experiments. Scanning electron microscopy (SEM) micrographs were taken with a JEOL model JSM-5400 scanning electron microscope operated at an accelerating voltage of 15 KV. Transmission electron microscopy (TEM) micrographs were taken with a JEOL model JEM-1010 transmission electron microscope operated at an accelerating voltage of 80 KV. Specimens for the TEM were prepared by casting one drop of the PbS NP dispersion (at a 1:10 dilution in water) onto standard Formvar/silicon monoxide-coated copper grids (400 mesh, Electron Microscopy Science). Excess liquid was wicked away with filter paper, and the grid was dried in air.

2.3. Electrochemical immunoassay (ECIA) detection scheme

The procedure is illustrated in the following electrochemical immunoassay (ECIA) detection scheme (cf. Fig. 1). Briefly, CEA is first captured using magnetic beads conjugated with the primary CEA antibody (MB–anti-CEA). The PbS-labeled secondary antibody (PbS–anti-CEA) is then introduced to form an immunocomplex sandwich on the magnetic-bead surface ($^{MB}Ab/Ag_{CEA}/Ab^{PbS}$). The reaction mixture is subjected to magnetic processing to eliminate the excess reagents and thereby purify the product. Then, the captured PbS NP labels are dissolved with 1.0 mol/l HCl to release lead ions. This step is followed by an additional magnetic separation step and square wave voltammetric (SWV) detection of the released lead ions on an *in situ*-plated mercury film electrode in a 0.2 mol/l acetate buffer (pH 4.6).

2.3.1. Preparation of reagents

Thioglycolic acid (TGA)-stabilized PbS nanoparticles were synthesized following the methodology reported in [31] with modifications (cf. Supporting information). The PbS–anti-CEA conjugate was prepared following the methodologies reported in [25,32] with modifications. Anti-CEA-coated magnetic bead (MB) conjugates were prepared following the methodologies reported in [25,32] with modifications.

2.3.2. Magnetic bead-based immunoassay

Experimental parameters, such as the amount of PbS–anti-CEA conjugate and the amount of MB–anti-CEA conjugate, were optimized prior to the assay according to the principles of PbS NP-based ECIA (Fig. 1). The magnetic bead-based immunoassay was performed in 1.5 ml centrifuge tubes on a biomagnetic processing platform according to the protocol established in [33] with modifications (cf. Supporting information).

2.4. Electrochemical measurements

The procedure for the *in situ*-plating of a mercury film on a SPCE was performed at room temperature using differential pulse voltammetry (DPV) according to the protocol established in [34] with modifications (cf. Supporting information). The potential applied for preconcentrating lead ions onto the SPCE/Hg was optimized according to the protocol established in [35] with modifications (cf. Supporting information). SWV measurements were performed with an SPCE consisting of a carbon working electrode (WE-C), a carbon counter electrode (AE-C) and a Ag/AgCl pseudo-reference electrode (RE-Ag). Prior to performing the electrochemical measurements, the SPCE was pretreated for 60 s in 0.05 mol/l PBS at a potential of +1.5 V to clean the electrode surface. After washing and drying with air, 50 μl of the sample solution was placed on the sensing area of the three electrodes to form an electrochemical cell. A sensor connector was used to connect the SPCE and the portable bipotentiostat μ-Stat, 200. The lead ions that were released from the immunocomplex sandwich were measured with an SWV using an *in situ* mercury-plated film on the SPCE, following a 65 s pretreatment at +0.6 V and accumulation for 120 s at −1.4 V. Subsequent square wave measurements were performed after a 5 s rest period from −1.0 to −0.4 V with a step potential of 4 mV, an amplitude of 25 mV and a frequency of 15 Hz. A baseline correction of the resulting voltammogram was performed using DropView software.

Validation and method comparison experiments were completed using a Carcinoembryonic antigen (CEA) Test System ($C_{CEA} = 0$, 1.0, 5.0, 10.0, 25.0, 50.0 and 250.0 ng/ml) that was purchased from AccuBind ELISA Microwells MonoBind® and used according to the manufacturer's instructions (cf. Supporting information). Note: Cross-reactivity against